PCT WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 5 :		(11) International Publicati n Number:
A61K 31/13, 31/14	A1	WO 93/20806
		(43) International Publication Date: 28 October 1993 (28.10.93)

(21) International Application Number: PCT/US93/02922

(22) International Filing Date: 29 March 1993 (29.03.93)

(30) Priority data:
867,759 13 April 1992 (13.04.92) US

(71) Applicant: THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; National Institutes of Health, Office of Technology Transfer, Box OTT, Bethesda, MD 20892-9902 (US).

(72) Inventors: MARAGOS, Chris, M. ; 805A Stratford Drive, Frederick, MD 21701 (US). WANG, Ji, Ming ; M302 Waverly Drive, Frederick, MD 21701 (US). KEEFER, Larry, K. ; 7016 River Road, Bethesda, MD 20817 (US). OPPENHEIM, Joost, J. ; 7601 Winterberry Place, Bethesda, MD 20817 (US).

(74) Agents: KILYK, John, Jr. et al.; Leydig, Voit & Mayer, Ltd., Two Prudential Plaza, Suite 4900, Chicago, IL 60601-6780 (US).

(81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

Published
With international search report.

(54) Title: USE OF NITRIC OXIDE/NUCLEOPHILE COMPLEXES FOR THE TREATMENT OF CANCER

(57) Abstract

A method of inducing cell cytostasis or cytotoxicity by exposing cells to a compound capable of releasing nitric oxide in an aqueous solution, particularly a nitric oxide/nucleophile complex or a derivative thereof, and a pharmaceutical composition containing a therapeutically effective amount of such a compound with a pharmaceutically acceptable carrier.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AT	Austria	FR	France	MR	Mauritania
AU	Australia	GA	Gabon	MW	Malawi
BB	Barbados	GB	United Kingdom	NL	Netherlands
BE	Belgium	GN	Guinea	NO	Norway
BF	Burkina Faso	GR	Greece	NZ	New Zealand
BG	Bulgaria	HU	Hungary	PL	Poland
BJ	Benin	IE	Ireland	PT	Portugal
BR	Brazil	IT	Italy	RO	Romania
CA	Canada	JP	Japan	RU	Russian Federation
CF	Central African Republic	KP	Democratic People's Republic of Korea	SD	Sudan
CG	Congo			SE	Sweden
CH	Switzerland	KR	Republic of Korea	SK	Slovak Republic
CI	Côte d'Ivoire	KZ	Kazakhstan	SN	Senegal
CM	Cameroon	LI	Liechtenstein	SU	Soviet Union
CS	Czechoslovakia	LK	Sri Lanka	TD	Chad
CZ	Czech Republic	LU	Luxembourg	TG	Togo
DE	Germany	MC	Monaco	UA	Ukraine
DK	Denmark	MG	Madagascar	US	United States of America
ES	Spain	ML	Mali	VN	Viet Nam
FI	Finland	MN	Mongolia		

USE OF NITRIC OXIDE/NUCLEOPHILE COMPLEXES FOR THE TREATMENT OF CANCER

TECHNICAL FIELD OF THE INVENTION

The present invention concerns a method of, and composition for, controlling the proliferation of cells. In particular, the present invention is directed to th use of compounds which release nitric oxide in aqueous solutions, particularly nitric oxide/nucleophil complexes and their derivatives, to induce cytostasis and cytotoxicity so as to attenuate cell proliferation.

BACKGROUND OF THE INVENTION

The control of cell proliferation has great usefulness in the treatment of various disorders. Of particular interest in that respect is the attenuation of cell growth or proliferation in the treatment of tumor or cancerous cells, parasites, fungi, bacteria, and other proliferating organisms.

Nitric oxide (NO) has recently been implicated in a variety of bioregulatory processes, including normal physiological control of blood pressure, macrophage-induced cytostasis and cytotoxicity, and neurotransmission (Moncada et al., "Nitric Oxide from L-Arginine: A Bioregulatory System," Excerpta Medica, International Congress Series 897 (Elsevier Science Publishers B.V.: Amsterdam, 1990); Marletta et al., "Unraveling the Biological Significance of Nitric Oxide," Biofactors, 2, 219-225 (1990); Ignarro, "Nitric Oxide. A Novel Signal Transduction Mechanism for Transcellular Communication," Hypertension (Dallas), 16, 477-483 (1990)). A number of compounds have been developed which are capable of delivering nitric oxide, including compounds which release nitric oxide upon being metabolized and compounds which release nitric oxide in aqueous solution.

Those compounds which release nitric oxide upon being metabolized include the widely used nitrovasodilators glyceryl trinitrate and sodium nitroprusside (Ignarro et al., J. Pharmacol. Exp. Ther., 218, 739-749 (1981); Ignarro, Annu. Rev. Pharmacol. Toxicol., 30, 535-560 (1990); Kruszyna et al., Toxicol. Appl. Pharmacol., 91, 429-438 (1987); Wilcox et al., Chem. Res. Toxicol., 3, 71-76 (1990)). Another compound, S-nitroso-N-acetylpenicillamine, has been reported to release nitric oxide in solution and as being effective at inhibiting DNA synthesis (Garg et al., Biochem. and Biophys. Res. Comm., 171, 474-479 (1990)).

Nitric oxide/nucleophile complexes which release nitric oxide in aqueous solution are disclosed in U.S. Patents 4,954,526 and 5,039,705, as well as in pending U.S. patent applications 07/423,279 (filed October 18, 1989), 07/585,793 (filed September 20, 1990), 07/743,892 (filed August 12, 1991), 07/764,906 (filed September 24, 1991), 07/764,908 (filed September 24, 1991), and 07/858,885 (filed March 27, 1992), as being useful cardiovascular agents (see also Maragos et al., J. Med. Chem., 34, 3242-3247 (1991)).

The cytostatic effect of nitric oxide solutions on tumor cells in vitro has been demonstrated. In particular, it has been shown that solutions of nitric oxide inhibit DNA synthesis and mitochondrial respiration of tumor cells in vitro (Hibbs et al., Biochem. and Biophys. Res. Comm., 157, 87-94 (1988); Stuehr et al., J. Exp. Med., 169, 1543-1555 (1989)). Nitric oxide in its pure form, however, is a highly reactive gas having limited solubility in aqueous media (WHO Task Group on Environmental Health Criteria for Oxides of Nitrogen, Oxides of Nitrogen, Environmental Health Criteria 4 (World Health Organization: Geneva, 1977)). Nitric oxide, therefore, is difficult t

introduce reliably int most biological systems without premature decompositi n.

The difficulty in administering nitric oxide can be overcome in some cases by administering nitric oxide pharmacologically in prodrug form. The compounds glyceryl trinitrate and sodium nitroprusside are relatively stable but release nitric oxide only on redox activation (Ignarro et al., J. Pharmacol. Exp. Ther., 218, 739-749 (1981); Ignarro, Annu. Rev. Pharmacol. Toxicol., 30, 535-560 (1990); Kruszyna et al., Toxicol. Appl. Pharmacol., 91, 429-438 (1987); Wilcox et al., Chem. Res. Toxicol., 3, 71-76 (1990)). While this feature may be an advantage in some applications, it can also be a significant liability, as in the development of tolerance to glyceryl trinitrate via the exhaustion of the relevant enzyme/cofactor system (Ignarro et al., Annu. Rev. Pharmacol. Toxicol., 25, 171-191 (1985); Kuhn et al., J. Cardiovasc. Pharmacol., 14(Suppl. 11), S47-S54 (1989)) and toxicity from metabolically produced cyanide during prolonged administration of nitroprusside (Smith et al., "A Potpourri of Biologically Reactive Intermediates" in Biological Reactive Intermediates IV, Molecular and Cellular Effects and Their Impact on Human Health (Witmer et al., eds.), Advances in Experimental Medicine and Biology Volume 283 (Plenum Press: New York, 1991), pp. 365-369).

There remains a need, therefore, for a method of inhibiting the proliferation of tumor cells, parasites, fungi, bacteria, and other proliferating cells or organisms which can be reliably effected in vivo and which preferably does not require undesirable activation mechanisms. It is an object of the present invention to provide such a method. It is another object of th present invention to provide a pharmaceutical composition useful in carrying out that method.

These and oth r objects and advantages of the present inventi n, as well as additional inventive

features, will be apparent from th description of th invention provided h r in.

BRIEF SUMMARY OF THE INVENTION

The present invention encompasses a method of controlling cell proliferation by inducing cytostasis and/or cytotoxicity among the cells. Specifically, the present invention involves exposing cells to a compound capable of releasing nitric oxide in an aqueous solution, particularly a nitric oxide/nucleophile complex or derivative thereof.

The present invention also encompasses a pharmaceutical composition comprising a pharmaceutically acceptable carrier and a therapeutically effective amount of a compound capable of releasing nitric oxide in an aqueous solution, particularly a nitric oxide/nucleophile complex or derivative thereof. The pharmaceutical composition will generally contain an amount of the nitric oxide releasing compound sufficient to induce cytostasis or cytotoxicity among cells exposed to the pharmaceutical composition and has particular utility in the treatment of tumor cells, as well as in antiparasitic, antifungal, and antibacterial treatments.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a graph depicting the attenuation of <u>in vitro</u> tumor cell proliferation as reflected by the percent relative ^{3}H-thymidine incorporation by a melanoma subline incubated with various concentrations of various nitric oxide/nucleophile complexes.

Figure 2 is a graph depicting the attenuation of <u>in vitro</u> tumor cell proliferation as reflected by the percent relative ^{3}H-thymidine incorporation by a melanoma subline incubated with various concentrations of the nitric oxide complex of 3-(n-propylamino)propylamine with and without the presence of the nitric oxide scavenger xymyoglobin.

DESCRIPTION OF THE PREFERRED EMBODIMENTS

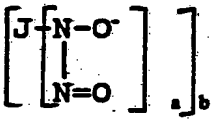
The present invention is predicated on the discovery that cell proliferation, particularly tumor cells proliferation, can be attenuated or inhibited by exposing cells to a compound that is capable of releasing nitric oxide in an aqueous solution, specifically a nitric oxide/nucleophile complex or a derivative thereof. The present invention concerns a method of controlling cell proliferation by exposing cells to a compound capable of releasing nitric oxide in an aqueous solution, as well as a pharmaceutical composition which includes a pharmaceutically acceptabl carrier and a therapeutically effective amount of a compound capable of releasing nitric oxide in an aqueous solution, particularly a nitric oxide/nucleophile complex or a derivative thereof.

The compounds that are capable of releasing nitric oxide in an aqueous solution in the context of the present invention preferably do so spontaneously upon contacting an aqueous environment, e.g., do not require activation through a redox reaction or electron transfer such as required for glyceryl trinitrate and sodium nitroprusside. Some of the nitric oxide/nucleophil complexes useful in the context of the present invention do require activation by particular means, but only as necessary to free the nitric oxide releasing $X[N(O)NO]^-$ group in the vicinity of the particular cells of interest. As an example, covalent attachment of a protecting group to the anionic $[N(O)NO]^-$ function provides a means of postpoining nitric oxide releas until the molecule reaches an organ capable of metabolically removing the protecting group. By choosing a protecting group that is selectively cleaved by tumor-specific enzymes, for example, the action of the nitric oxide/nucleophile complex can be targeted to maximize the desired effect. While the nitric oxide releasing compound in the context of the present

invention is capable of rel asing nitric oxide in an aqueous solution, such a compound preferably releases nitric oxid under physiological conditions.

The compound capable of releasing nitric oxide in an aqueous solution is preferably a nitric oxide/nucleophile adduct, e.g., a complex of nitric oxide and a nucleophile, most preferably a nitric oxide/nucleophile complex which contains the anionic moiety $X[N(O)NO]^-$, where X is any suitable nucleophil residue. The nucleophile residue is preferably that of a primary amine (e.g., X = $(CH_3)_2CHNH$, as in $(CH_3)_2CHNH[N(O)NO]Na$), a secondary amine (e.g., X = $(CH_3CH_2)_2N$, as in $(CH_3CH_2)_2N[N(O)NO]Na$), a polyamine (e.g., X = spermine, as in the zwitterion $H_2N(CH_2)_3NH_2^+(CH_2)_4N[N(O)NO]^-(CH_2)_3NH_2$, or X = 3-(n-propylamino) propylamine, as in the zwitterion $CH_3CH_2CH_2N[N(O)NO]^-CH_2CH_2CH_2NH_3^+$), or oxide (i.e., X = O^-, as in $NaO[N(O)NO]Na$), or a derivative thereof. Such nitric oxide/nucleophile complexes are stable solids and are capable of delivering nitric oxide in a biologically usable form at a predictable rate. The nucleophile is preferably not an entity such as sulfite (e.g., X = SO_3^-, as in $NH_4O_3S[N(O)NO]NH_4$) even though the complex is a stable compound, since it is capable of releasing nitric oxide in an aqueous environment only under harsh, nonphysiological conditions.

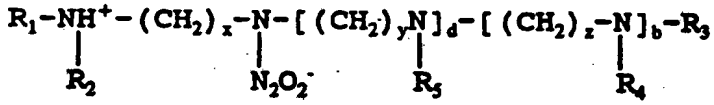
Other suitable nitric oxide/nucleophile complexes include those having the following formulas:



$$M_c^{+x} \qquad \text{(I)}$$

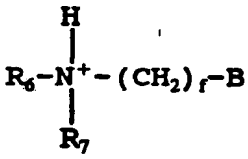
wherein J is an organic or inorganic moiety, preferably a moiety which is not linked to the nitrogen of the remainder of the complex through a carbon atom, M^{+x} is a pharmaceutically acceptabl cation, where x is th valence of the cation, a is 1 or 2, and b and c are the smallest integers that result in a neutral compound,

preferably such that the compound is n t a salt f alanosin or d pastin;

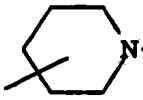


(II)

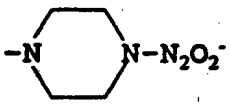
wherein b and d are the same or different and may be zero or one, R_1, R_2, R_3, R_4, and R_5 are the same or different and may be hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-trichloro-t-butoxycarbonyl, and x, y, and z are the same or different and are integers from 2 to 12, with the proviso that R_1 and R_3 are preferably not both methyl when R_2 is hydrogen, x is 2, and b and d are zero;



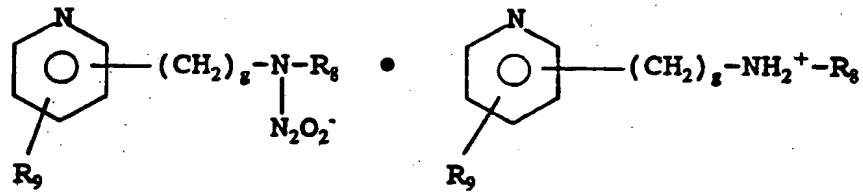
(III)

wherein B is  or  ,

R_6 and R_7 are the same or different and may be hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-trichloro-t-butoxycarbonyl, f is an integer from 0 to 12, with the proviso that when B is the substituted piperazine moiety

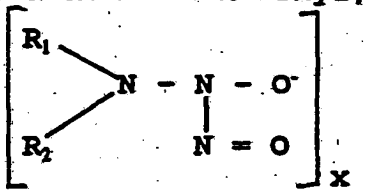


then f is an integer from 2 to 12;



(IV)

wherein R_8 is hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-tri-chloro-t-butoxycarbonyl, R_9 is hydrogen or a C_1-C_{12} straight or branched chain alkyl, and g is 2 to 6;



(V)

wherein R_1 and R_2 are independently selected from the group consisting of a straight chain or branched chain C_1 - C_{12} alkyl group and a benzyl group, with the proviso that no branch occur on the alpha carbon atom, or else R_1 and R_2 together with the nitrogen atom they are bonded to form a heterocyclic group, M^{+x} is a pharmaceutically acceptable cation, and x is the valence of the cation;

$$K[(M)^{x'}_{x}(L)_y(R^1R^2N\text{-}N_2O_2)_z] \qquad \text{(VI)}$$

wherein M is a pharmaceutically acceptable metal, or where x is at least two, a mixture of two different pharmaceutically acceptable metals, L is a ligand different from $(R^1R^2N\text{-}N_2O_2)$ and is bound to at least one metal, R^1 and R^2 are each organic moieties and may be the same or different (with the proviso that where M is copper, x is one, L is methanol, and y is one, that at least one of R^1 or R^2 is not ethyl), x is an integer of from 1 to 10, x' is the formal oxidation state of the metal M, and is an integer of from 1 to 6, y is an integer of from 1 to 18, and where y is at least 2, the ligands L may be the same or different, z is an integer of from 1 to 20, and K is a pharmaceutically acceptable counterion to render the compound neutral to the extent necessary; and

$$[R\text{-}N(H)N(NO)O\text{-}]_yX \qquad \text{(VII)}$$

wherein R is C_{2-8} lower alkyl, phenyl, benzyl, or C_{3-8} cycloalkyl, any of which R groups may be substituted by one to three substituents, which are the same or

different, selected from the group consisting of hal , hydroxy, C_{1-8} alkoxy, $-NH_2$, $-C(O)NH_2$, -CH(O), -C(O)OH, and $-NO_2$, X is a pharmaceutically acceptabl cati n, a pharmaceutically acceptable metal center, or a pharmaceutically acceptable organic group selected from the group consisting of C_{1-8} lower alkyl, $-C(O)CH_3$, and $-C(O)NH_2$, and y is one to three, consistent with th valence of X.

In addition to the above-recited compounds, pharmaceutically acceptable salts, zwitterions, and derivatives thereof are also useful in the context f the present invention.

The synthesis of nitric oxide/nucleophile complexes generally involves reacting nitric oxide with suitabl nucleophiles and has been described in Drago, "Reactions of Nitrogen(II) Oxide," in Free Radicals in Inorganic Chemistry, Advances in Chemistry Series, Number 36 (American Chemical Society: Washington, DC, 1962), pp. 143-149; Hansen et al., "N-Nitrosation of Secondary Amines by Nitric Oxide via the 'Drago Complex,'" in N-Nitroso Compounds: Occurrence and Biological Effects (Bartsch et al., eds.), IARC Scientific Publications No. 41 (Int'l Agency for Research on Cancer: Lyon, 1982), pp. 21-29; and Maragos et al., J. Med. Chem., 34, 3242-3247 (1991).

The rate at which the nitric oxide/nucleophile complex releases nitric oxide is dependent on at least the pH of the aqueous solution, the temperature, and th specific nature of the nucleophile. In general, the more alkaline the medium and the lower the temperature, the slower the release of nitric oxide. The nature of the nucleophile influences the rate of nitric oxide release over a considerable range (as demonstrated in the Examples herein). The effect on cell proliferation of th compound capabl of releasing nitric oxide, therefore, can be controlled by appropriate sel ction of the nitric oxide releasing compound.

The effect of a compound capable of releasing nitric oxide in an aqueous solution on cells is reversible in the sense that the addition of a compound which is capable of removing or scavenging nitric oxide from an aqueous solution by complexing or reacting with nitric oxide can counteract the inhibitory effect of the compound which releases the nitric oxide. The effect on cell proliferation of the compound capable of releasing nitric oxide, therefore, can be further controlled by use of such a nitric oxide scavenger compound in an appropriate quantity.

While the method of the present invention can be practiced in vitro, it has particular usefulness in in vivo applications. The present inventive method, therefore, includes the administration to an animal, particularly a human, of a therapeutically effective amount of a compound capable of releasing nitric oxide in an aqueous solution, particularly a nitric oxide/nucleophile complex or derivative thereof. The use of such a compound in treating animals, particularly humans, circumvents the disadvantages of the use of pure nitric oxide, aqueous solutions of nitric oxide, and compounds which release nitric oxide but require undesirable activation mechanisms.

As regards the in vivo use of the present inventive method, a nitric oxide releasing compound in the context of the present invention can be administered in any suitable manner, preferably with pharmaceutically acceptable carriers. The attenuation of tumor cell proliferation in an animal has utility in, for example, the in vivo treatment of cancerous cells.

One skilled in the art will appreciate that suitable methods of administering a nitric oxide releasing compound in the context of the present invention to an animal are available, and, although more than one route can be used to administer a particular compound, a particular route can provide a more

immediat and mor effectiv reaction than another route. Pharmaceutically acc ptable carri rs are also well-kn wn to those who are skilled in th art. Th choice of carrier will be determined in part by the particular compound, as well as by the particular method used to administer the composition. Accordingly, ther is a wide variety of suitable formulations of the pharmaceutical composition of the present invention.

Formulations suitable for oral administration can consist of (a) liquid solutions, such as an effectiv amount of the compound dissolved in diluents, such as water or saline, (b) capsules, sachets or tablets, each containing a predetermined amount of the active ingredient, as solids or granules, (c) suspensions in an appropriate liquid, and (d) suitable emulsions. Tablet forms can include one or more of lactose, mannitol, corn starch, potato starch, microcrystalline cellulose, acacia, gelatin, colloidal silicon dioxide, croscarmellose sodium, talc, magnesium stearate, stearic acid, and other excipients, colorants, diluents, buffering agents, moistening agents, preservatives, flavoring agents, and pharmacologically compatible carriers. Lozenge forms can comprise the activ ingredient in a flavor, usually sucrose and acacia or tragacanth, as well as pastilles comprising the active ingredient in an inert base, such as gelatin and glycerin or sucrose and acacia emulsions, gels, and th like containing, in addition to the active ingredient, such carriers as are known in the art.

The nitric oxide releasing compounds in the context of the present invention, alone or in combination with other suitable components, can be made into aerosol formulations to be administered via inhalation. These aerosol formulations can be placed into pressurized acceptable propellants, such as dichlorodifluoromethane, propane, nitrogen, and th like.

Formulations suitable for parenteral administration include aqueous and non-aqueous, isotonic sterile injection solutions, which can contain anti-oxidants, buffers, bacteriostats, and solutes that render the formulation isotonic with the blood of the intended recipient, and aqueous and non-aqueous sterile suspensions that can include suspending agents, solubilizers, thickening agents, stabilizers, and preservatives. The formulations can be presented in unit-dose or multi-dose sealed containers, such as ampules and vials, and can be stored in a freeze-dried (lyophilized) condition requiring only the addition of the sterile liquid carrier, for example, water, for injections, immediately prior to use. Extemporaneous injection solutions and suspensions can be prepared from sterile powders, granules, and tablets of the kind previously described.

The dose administered to an animal, particularly a human, in the context of the present invention should be sufficient to effect a therapeutic response in the animal over a reasonable time frame. The dose will be determined by the strength of the particular compound employed and the condition of the animal, as well as the body weight of the animal to be treated. The size of the dose also will be determined by the existence, nature, and extent of any adverse side-effects that might accompany the administration of a particular compound.

In the treatment of some individuals with the pharmaceutical composition of the present invention, it may be desirable to utilize a "mega-dosing" regimen. In such a treatment, a large dose of the pharmaceutical composition is administered to an individual, time is allowed for the active compound, i.e., the nitric oxide releasing compound, to act, and then a suitable reagent, e.g., a nitric oxide scavenger, is administered to the individual to render the active compound ineffective.

The desirable extent of the inhibition of cell proliferation rate will depend on the particular condition or disease being treated, as well as the stability of the patient and possible side-effects. In proper doses and with suitable administration of certain compounds, the present invention provides for a wide range of the inhibition of the normal cell proliferation rate, e.g., from little inhibition to essentially full inhibition.

The cytostatic activity of a nitric oxide/nucleophile complex is generally related to the rate and extent of nitric oxide release. It is believed that those compounds which release nitric oxide slowly, such as spermine-bis(nitric oxide) adduct monohydrate and 3(n-propylamino)propylamine bis(nitric oxide) adduct, are more potent inhibitors of DNA synthesis than compounds which release nitric oxide more quickly, such as diethylamine-bis(nitric oxide) adduct sodium salt, isopropylamine-bis(nitric oxide) adduct sodium salt, and sodium trioxodinitrate(II) monohydrate (also known as "Angeli's salt"). It is also believed that a sustained exposure to moderate amounts of nitric oxide has a longer lasting effect than a brief exposure to a greater concentration of nitric oxide. Moreover, the number of molecules of nitric oxide released by a single nitric oxide/nucleophile complex, which will generally vary up to two per [N(O)NO] moiety, apparently affects the potency of the nitric oxide/nucleophile complex, with compounds which release more nitric oxide per molecule having a greater cytostatic effect.

The cytostatic effect of a nitric oxide/nucleophile complex is also dependent on factors in addition to the rate and extent of nitric oxide release. Such factors include the mechanism by which the compound degrades, the degree of uptake by the exposed cells, and the affinity for cellular constituents. For example, spermine binds DNA which may play a role in the nitric

oxide/spermine complex having a high cytostatic effect on tum r cells.

While the present inventive method and pharmaceutical composition are primarily directed to the control of tumor cell proliferation, i.e., cytostasis or the inhibition of cell division, the present inventiv method and pharmaceutical composition may also have usefulness in controlling tumor cell growth, i.e., by providing a cytotoxic effect to adversely affect cell viability, and in attenuating the proliferation and growth of non-tumor cells, e.g., as antiparasitic, antifungal, and antibacterial agents, as well as in laboratory research. Those compounds which have a cytostatic effect but not a cytotoxic effect can be used with cytotoxic agents in combination therapy. The present invention also has usefulness in prophylatic treatments.

Some of the nitric oxide releasing compounds, in sufficiently high concentration, will have a cytotoxic effect, in addition to a cyctostatic effect, on cells. For example, under at least the conditions set forth in the Exaples below, spermine-bis(nitric oxide) adduct monohydrate has a pronounced cytotoxic effect at a concentration of 500 μM, while 3-(n-propylamino)propylamine bis(nitric oxide) adduct, diethylamine-bis(nitric oxide) adduct sodium salt, isopropylamine-bis(nitric oxide) adduct sodium salt, sodium trioxodinitrate(II) monohydrate, and N-nitrosohydroxylamine-N-sulfonate do not significantly affect cell viability at concentrations up to 500 μM. The cytotoxic effect of a particular nitric oxide/nucleophile complex is generally related to the nature of the nucleophile and other chemical entities which may be released from the complex, e.g., spermine from the spermine-nitric oxide complex.

The following examples further illustrate the present invention but, of course, should not be construed as in any way limiting its scope.

Example 1

The effect on tumor cell proliferation by various nitric oxide/nucleophile complexes was evaluated by examining the effectiveness of such complexes in inhibiting DNA synthesis in A375 human melanoma cells as measured by incorporation of tritiated thymidine. Specifically, A375-C6 human melanoma cells were exposed to 0, 25, 50, 100, 200, 300, and 500 μM of various nitric oxide/nucleophile complexes.

A375-C6 human melanoma cells were harvested with trypsin, washed twice with 10 mM phosphate buffered saline (PBS), and resuspended in RMPI 1640 medium (Advanced Biotechnologies, Inc., Columbia, Maryland) containing 5% fetal bovine serum (Hyclone) supplemented with 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were diluted to 5.3 x 10^4/ml and 0.95 ml aliquots were distributed into microfuge tubes. Fifty microliters of each nitric oxide/nucleophile complex in 100 mM PBS, or PBS control, were added and mixed vigorously.

The cell suspensions were distributed onto flat bottom tissue culture clusters (Costar) in the volume of 0.2 ml/well. Tritiated thymidine stock, i.e., ^{3}H-thymidine (^{3}H-TdR) (NEN, Boston, Massachusetts), with a specific activity of 15.0 Ci/mmol, was diluted in medium and added at a level of 1 μCi/well (25 μl/well). Following an 8 hour incubation, at 37°C and 5% Co_2, the cell contents were collected onto filter discs (Model 290 PHD Cell Harvester, Cambridge Technology, Inc., Watertown, Massachusetts). The radioactivity of the filters was measured (Beckman LS-900) after the addition of 4 ml scintillation cocktail (Aquassure,, NEN). DNA synthesis was expressed as % relative incorporation of

labeled thymidine, i.e., (cpm sample/cpm PBS control) x 100%.

The perc nt relativ ^{3}H-TdR incorporations versus the concentration of the nitric oxide/nucleophile complexes were plotted as shown in Figure 1, and curves were plotted for the data points for equations fitted to each data set with the aid of a commercial computer program (TableCurve, Jandel Scientific, Corte Madera, California). The data were fit to a dose-response equation of the form:

% Relative Incorporation = $a + b/(1 + (Dose/c)^d)$.

This equation is equivalent to the sigmoid E_{max} model with baseline correction as described in Holford et al., Clin. Pharmacokinetics, 6, 429-453 (1981).

Each data point in Figure 1 represents the mean of the results for three separate experiments, with 3-6 wells being used for each experiment (i.e., n = 9-18 wells per data point). The bars represent 95% confidence intervals based on an evaluation of th standard deviation of the experimental results for each data point.

The following nitric oxide/nucleophile complexes were tested, and the concentration of each of the complexes required to inhibit ^{3}H-TdR incorporation by 50% (IC_{50}) was estimated from the equations fit to each data set used to prepare the curves of Figure 1.

Compound	Data Point Symbol	IC_{50} (mean ± std. dev., μM)
$H_2N(CH_2)_3NH_2^+(CH_2)_4N[N(O)NO]^-(CH_2)_3NH_2$	●	24 ± 8
$CH_3CH_2CH_2N[N(O)NO]^-CH_2CH_2CH_2NH_3^+$	▲	44 ± 12[a]
$(CH_3CH_2)_2N[N(O)NO]Na$	▲	128 ± 6
$NaO[N(O)NO]Na$	○	287 ± 51
$(CH_3)_2CHNH[N(O)NO]Na$	■	> 500[b]
$NH_4(O_3S)[N(O)NO]NH_4$	□	NR[c]

[a] IC_{50} value represents mean of experimental results depicted in Figure 1 as well as from three additional experiments.

[b] Thymidine incorporation was inhibited by 43% at 500 μM (% relative inhibition = 57%).

[c] The term "NR" indicated no response and means that the compound did not inhibit DNA synthesis at 500 μM.

Controls, all tested concurrently with the nitric oxide/nucleophile complexes at a level of 500 μM, gav the following responses, where n represents the number of wells per data point:

Compound	n	percent relative thymidine incorporation (mean ± std. dev.)
isopropylamine	18	104 ± 15%
sodium nitrite	18	100 ± 16%
diethylamine	18	106 ± 21%
N-nitrosodiethylamine	18	95 ± 10%
3-(n-propylamino)propylamine	35	97 ± 14%
spermine	18	51 ± 13%

For comparison purposes, pure nitric oxide, added as 53 μl of a saturated solution to 950 μl cells (final concentration of 100 μM) resulted in a decrease in thymidine incorporation which was intermediate between the decrease in thymidine incorporation observed for $Na_2N_2O_3$ and $(C_2H_5)_2N[N(O)NO]Na$. The mean and standard deviation of percent relative incorporation for the pure nitric oxide were 72 ± 15% relative incorporation.

The results of this experiment demonstrate the inhibitory effect of nitric oxide/nucleophile complexes which release nitric oxide in aqueous solution on the proliferation of tumor cells.

Example 2

The effect on tumor cell proliferation by exposure to a nitric oxide/nucleophile complex in the presence of a compound which removes nitric oxide from an aqueous solution was evaluated by examining the effectiveness of the nitric oxide complex of 3-(n-propylamino)propylamine in inhibiting DNA synthesis in A375 human melanoma cells, by itself and in the presence of oxymyoglobin (MbO_2), as measured by incorporation of tritiated thymidine as s t out in Example 1.

A375-C6 human melanoma cells were xposed at 0, 20, 60, 100, 140, 170, and 200 μM of the nitric oxide complex of 3-(n-propylamino)propylamine in accordance with the procedure set out in Example 1, except that 6.3 x 10^4 cells in 0.90 ml were incubated with 0.05 ml, of 2.5 mM myoglobin (125 μM final concentration after dilution) or PBS control and 0.05 ml of the nitric oxide/nucleophile complex. Those cells exposed to oxymyoglobin were exposed to the oxymyoglobin immediately prior to the addition of the nitric oxide/nucleophile complex. The percent relative ^{3}H-TdR incorporations versus the concentration of the nitric oxide/nucleophile complex were plotted as shown in Figure 2, and curves were plotted for the data points for equations fitted to each data set utilizing the procedure set out in Example 1.

Each data point in Figure 2 represents the mean of the results for three separate experiments, with 3-6 wells being used for each experiment (i.e., n = 9-18 wells per data point). The bars represent 95% confidence intervals based on an evaluation of the standard deviation of the experimental results for each data point.

The concentration of the nitric oxide/nucleophile complex required to inhibit ^{3}H-TdR incorporation by 50% (IC_{50}) was estimated from the equations fit to each data set used to prepare the curves of Figure 2.

Presence of Oxymyoglobin	Data Point Symbol	IC_{50} (mean, μM)
oxymyoglobin present	o	NR[4]
oxymyoglobin not present	•	69

* The term "NR" indicated no response and means that the nitric oxide/nucleophile complex did n t inhibit DNA synthesis at 200 μM.

The results of this experiment demonstrate that a compound which removes nitric oxide from an aqueous solution, such as oxymyoglobin which forms a nitrosyl-myoglobin (Mb-NO) complex, provides a protective effect against the inhibitory effect on tumor cells of a compound which releases nitric oxide in an aqueous solution.

All of the references, including literature references, patents, and patent applications, cited herein are hereby incorporated in their entireties by reference.

While this invention has been described with an emphasis upon a preferred embodiment, it will be obvious to those of ordinary skill in the art that variations in the preferred method and composition may be used and that it is intended that the invention may be practiced otherwise than as specifically described herein. Accordingly, this invention includes all modifications encompassed within the spirit and scope of the invention as defined by the following claims.

WHAT IS CLAIMED IS:

1. A method of controlling tumor cell proliferation, which method comprises exposing tumor cells to a compound capable of releasing nitric oxide in an aqueous solution.

2. The method of claim 1, wherein said compound is capable of releasing nitric oxide under physiological conditions.

3. The method of claim 2, wherein said compound is a nitric oxide/nucleophile complex or a derivative thereof.

4. The method of claim 3, wherein said compound is a nitric oxide/nucleophile complex.

5. The method of claim 4, wherein said compound contains the anionic moiety $X[N(O)NO]^-$, where X is a nucleophile residue.

6. The method of claim 5, wherein X is selected from the group consisting of spermine, 3-(n-propylamino)propylamine, diethylamine, isopropylamine, and oxide.

7. The method of claim 3, wherein said compound is $(CH_3)_2CHNH[N(O)NO]Na$.

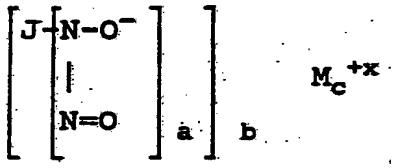
8. The method of claim 3, wherein said compound is $CH_3CH_2CH_2N[N(O)NO]^-CH_2CH_2CH_2NH_3^+$.

9. The method of claim 3, wherein said compound is $(CH_3CH_2)_2N[N(O)NO]Na$.

10. The method of claim 3, wherein said compound is $H_2N(CH_2)_3NH_2^+(CH_2)_4N[N(O)NO]^-(CH_2)_3NH_2$.

11. The method of claim 3, wherein said compound is NaO[N(O)NO]Na.

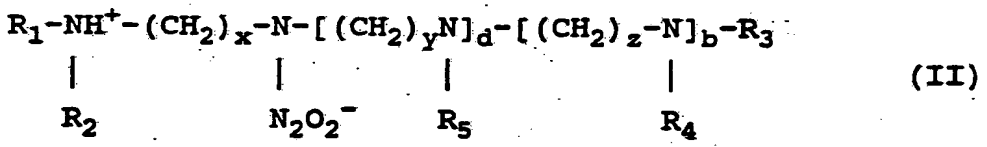
12. The method of claim 3, wherein said compound is of the formula I



(I)

wherein J is an organic or inorganic moiety, preferably a moiety which is not linked to the nitrogen of the remainder of the complex through a carbon atom, M^{+x} is a pharmaceutically acceptable cation, where x is the valence of the cation, a is 1 or 2, and b and c are the smallest integers that result in a neutral compound, or pharmaceutically acceptable salts or derivatives thereof.

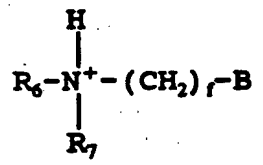
13. The method of claim 3, wherein said compound is of the formula II



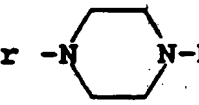
(II)

wherein b and d are the same or different and may be zero or one, R_1, R_2, R_3, R_4, and R_5 are the same or different and may be hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-trichloro-t-butoxycarbonyl, and x, y, and z are the same or different and are integers from 2 to 12, or pharmaceutically acceptable salts or derivatives thereof.

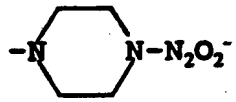
14. The method of claim 3, wherein said compound is of the formula III



(III)

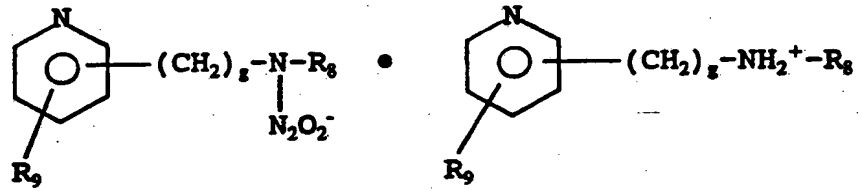
wherein B is  or  ,

R_6 and R_7 are the same or different and may be hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-trichloro-t-butoxycarbonyl, f is an integer from 0 to 12, with the proviso that when B is the substituted piperazine moiety



then f is an integer from 2 to 12, or pharmaceutically acceptable salts or derivatives thereof.

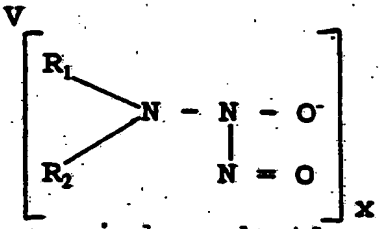
15. The method of claim 3, wherein said compound is of the formula IV



(IV)

wherein R_8 is hydrogen, C_{3-8} cycloalkyl, C_{1-12} straight or branched chain alkyl, benzyl, benzoyl, phthaloyl, acetyl, trifluoroacetyl, p-toluyl, t-butoxycarbonyl, or 2,2,2-tri-chloro-t-butoxycarbonyl, R_9 is hydrogen or a C_1-C_{12} straight or branched chain alkyl, and g is 2 to 6, or pharmaceutically acceptabl salts or derivatives thereof.

16. Th method of claim 3, wherein said comp und is f the formula V



(V)

wherein R_1 and R_2 are independently selected from the group consisting of a straight chain or branched chain C_1 - C_{12} alkyl group and a benzyl group, with the proviso that no branch occur on the alpha carbon atom, or else R_1 and R_2 together with the nitrogen atom they are bonded to form a heterocyclic group, M^{+x} is a pharmaceutically acceptable cation, and x is the valence of the cation, or pharmaceutically acceptable salts or derivatives thereof.

17. The method of claim 3, wherein said compound is of the formula VI

$$K[(M)^{x'}_{x}(L)_y(R^1R^2N\text{-}N_2O_2)_z] \qquad \text{(VI)}$$

wherein M is a pharmaceutically acceptable metal, or where x is at least two, a mixture of two different pharmaceutically acceptable metals, L is a ligand different from $(R^1R^2N\text{-}N_2O_2)$ and is bound to at least one metal, R^1 and R^2 are each organic moieties and may be the same or different (with the proviso that where M is copper, x is one, L is methanol, and y is one, that at least one of R^1 or R^2 is not ethyl), x is an integer of from 1 to 10, x' is the formal oxidation state of the metal M, and is an integer of from 1 to 6, y is an integer of from 1 to 18, and where y is at least 2, the ligands L may be the same or different, z is an integer of from 1 to 20, and K is a pharmaceutically acceptable counterion to render the compound neutral to the extent necessary, or pharmaceutically acceptabl salts or derivatives thereof.

18. The method of claim 3, wherein said compound is of the formula VII

$$[R\text{-}N(H)N(NO)O\text{-}]_yX \qquad \text{(VII)}$$

wherein R is C_{2-8} lower alkyl, phenyl, benzyl, or C_{3-8} cycloalkyl, any of which R groups may be substituted by one to three substituents, which are the same r different, selected from the group consisting of halo, hydroxy, C_{1-8} alkoxy, $-NH_2$, $-C(O)NH_2$, $-CH(O)$, $-C(O)OH$, and $-NO_2$, X is a pharmaceutically acceptable cation, a pharmaceutically acceptable metal center, or a pharmaceutically acceptable organic group selected from the group consisting of C_{1-8} lower alkyl, $-C(O)CH_3$, and $-C(O)NH_2$, and y is one to three, consistent with the valence of X, or pharmaceutically acceptable salts or derivatives thereof.

19. The method of claim 3, wherein said tum r cells are exposed to said nitric oxide releasing compound in an amount sufficient to inhibit the proliferation of said tumor cells.

20. The method of claim 3, wherein said compound is administered in a therapeutically effective amount with a pharmaceutically acceptable carrier to an animal.

21. The method of claim 20, wherein said animal is a human.

22. The method of claim 2, which method further comprises exposing said tumor cells to a compound capable of removing nitric oxide from an aqueous solution by complexing or reacting with nitric oxide so as to counteract the inhibitory effect of said compound capable of releasing nitric oxide in an aqueous solution.

23. A method of inducing cytostasis or cytotoxicity, which method comprises xposing cells to a compound capable of releasing nitric oxide in an aqueous solution in an amount sufficient to inhibit the proliferation of said cells.

24. The method of claim 23, wherein said compound induces cytostasis.

25. The method of claim 24, wherein said compound is capable of releasing nitric oxide under physiological conditions.

26. The method of claim 25, wherein said compound is a nitric oxide/nucleophile complex or a derivative thereof.

27. A pharmaceutical composition comprising a pharmaceutically acceptable carrier and a compound capable of releasing nitric oxide in an aqueous solution in a therapeutically effective amount sufficient to inhibit the proliferation of cells.

28. The pharmaceutical composition of claim 27, wherein said compound is capable of releasing nitric oxide under physiological conditions.

29. The pharmaceutical composition of claim 28, wherein said compound is a nitric oxide/nucleophile complex or a derivative thereof.

30. The pharmaceutical composition of claim 29, wherein said compound is a nitric oxide/nucleophile complex.

31. Th pharmaceutical composition of claim 30, wherein said compound contains the anionic moiety $X[N(O)NO]^-$, where X is a nucleophile residue.

32. The pharmaceutical composition of claim 31, wherein X is selected from the group consisting of spermine, 3-(n-propylamino)propylamine, diethylamin , isopropylamine, and oxide.

33. The method of claim 12, wherein said compound is not a salt of alanosine or dopastin.

34. The method of claim 13, wherein R_1 and R_3 are not both methyl when R_2 is hydrogen, x is 2, and b and d are zero.

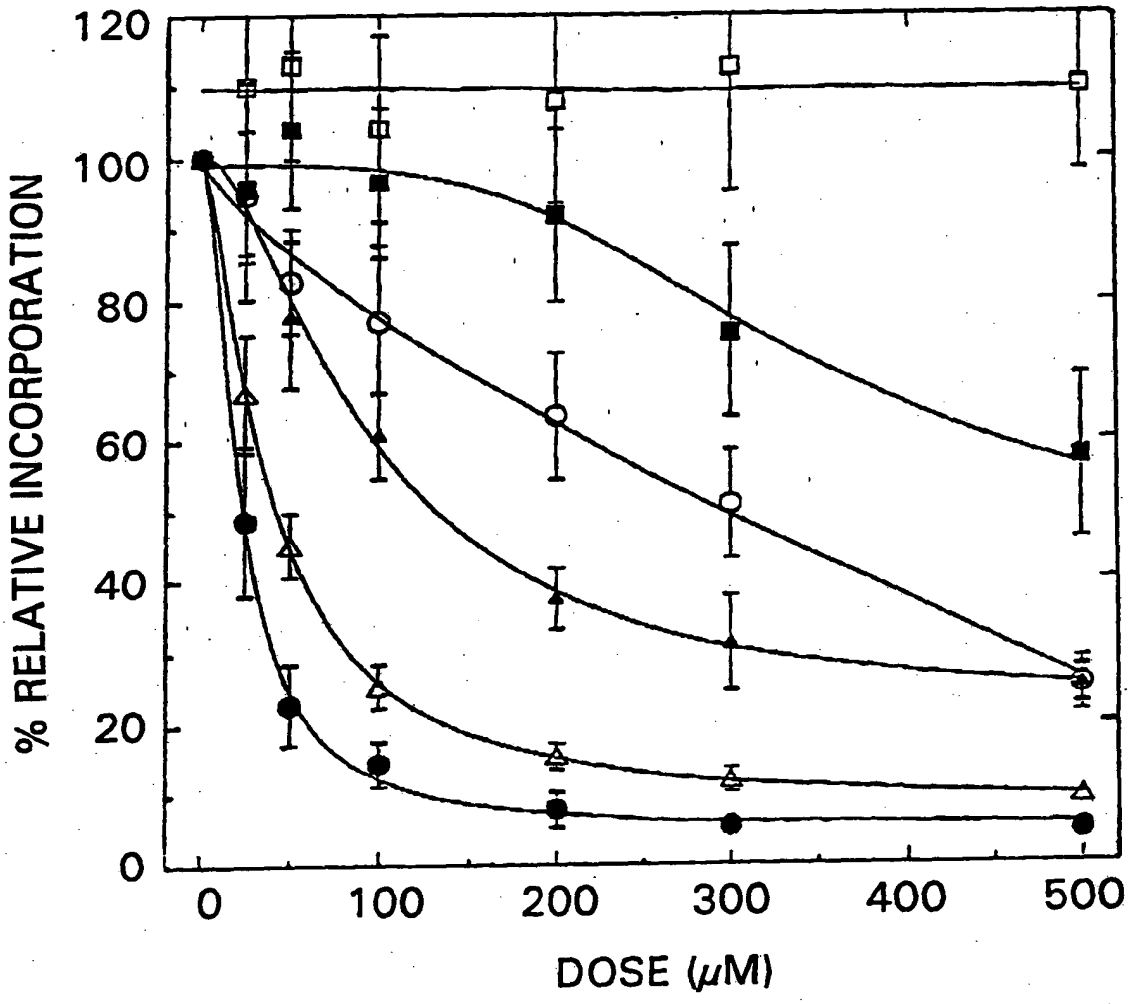


FIG. 1

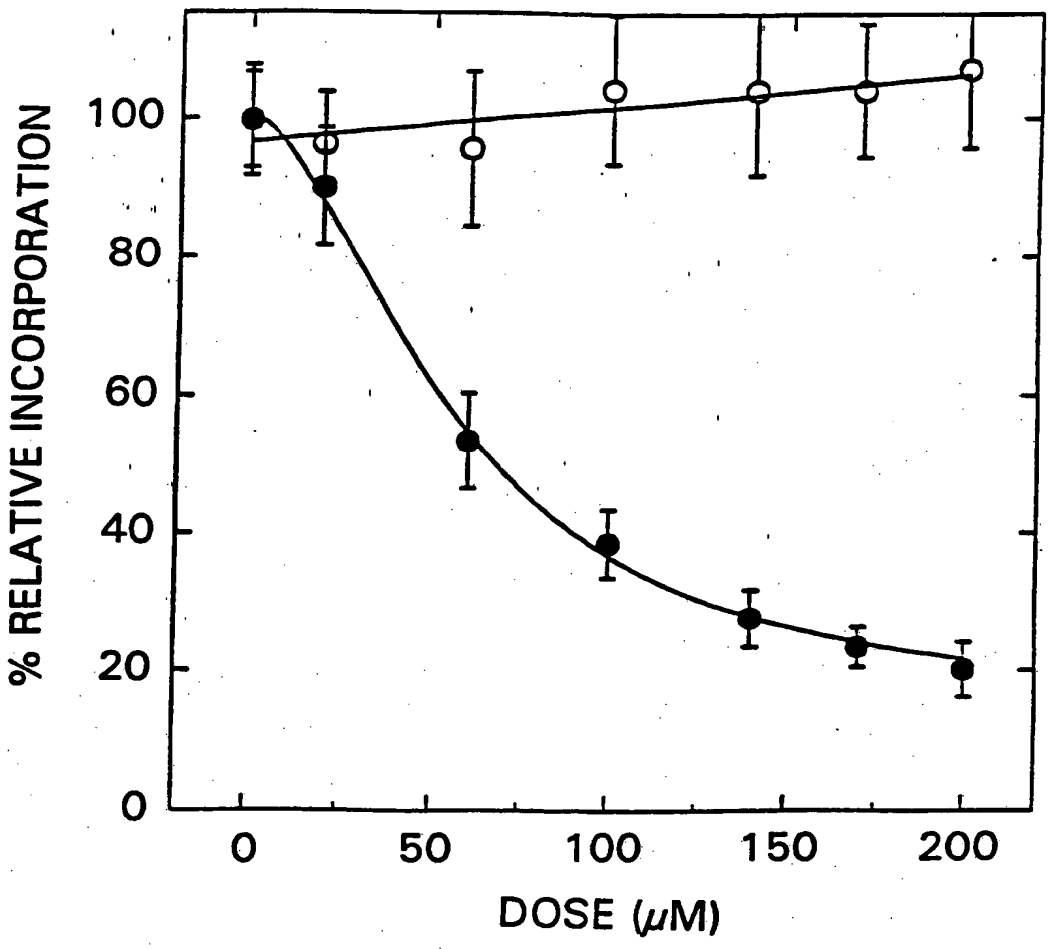


FIG. 2

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 93/02922

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all)[6]

According to International Patent Classification (IPC) or to both National Classification and IPC

Int.Cl. 5 A61K31/13; A61K31/14

II. FIELDS SEARCHED

Minimum Documentation Searched[7]

Classification System	Classification Symbols
Int.Cl. 5	A61K

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched[8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT[9]

Category °	Citation of Document,[11] with indication, where appropriate, of the relevant passages[12]	Relevant to Claim No.[13]
P,X	CANCER RESEARCH vol. 53, no. 3, 1 February 1993, pages 564 - 568 MARAGOS, C.M. ET AL 'NITRIC OXIDE/NUCLEOPHILE COMPLEXES INHIBIT THE IN VITRO PROLIFERATION OF A375 MELANOMA CELLS VIA NITRIC OXIDE RELEASE' see the whole document	1-11, 19-32
Y	JOURNAL OF MEDICINAL CHEMISTRY vol. 34, no. 11, November 1991, pages 3242 - 3247 MARAGOS, C.M. ET AL 'COMPLEXES OF .NO WITH NUCLEOPHILES AS AGENTS FOR THE CONTROLLED BIOLOGICAL RELEASE OF NITRIC OXIDE VASORELAXANT EFFECTS' cited in the application	1-7, 9-11, 19-21, 23-26
X	see the whole document	27-32

-/--

° Special categories of cited documents :[10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report.
06 JULY 1993	20.07.93
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	MAIR J.

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
A	SCIENCE vol. 254, 15 November 1991, pages 1001 - 1003 WINK, D.A. ET AL 'DNA DEAMINATING ABILITY AND GENOTOXICITY OF NITRIC OXIDE AND ITS PROGENITORS' see the whole document	1-34
X	THE JOURNAL OF BIOLOGICAL CHEMISTRY vol. 260, no. 7, 10 April 1985, pages 4069 - 4074 ALSTON,T. A. ET AL 'GENERATION OF NITRIC OXIDE BY ENZYMATIC OXIDATION OF N-HYDROXY-N-NITROSAMINES' see the whole document	1-5,12, 19-21, 23-31
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE vol. 169, no. 5, 1 May 1989, pages 1543 - 1555 STUEHR, D.J. ET AL 'NITRIC OXIDE A MACROPHAGE PRODUCT RESPONSIBLE FOR CYTOSTASIS AND RESPIRATORY INHIBITION IN TUMOR TARGET CELLS' cited in the application see the whole document	1-26,33, 34
Y	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS vol. 157, no. 1, 30 November 1988, pages 87 - 94 HIBBS, J.B. ET AL 'NITRIC OXIDE: A CYTOTOXIC ACTIVATED MACROPHAGE EFFECTOR MOLECULE' cited in the application see the whole document	1-21, 23-26, 33,34
Y	WO,A,9 009 785 (THE UNITED STATES OF AMERICA) 7 September 1990 cited in the application	1-7, 18-26
X	see the whole document	27-32
Y	WO,A,9 104 022 (THE UNITED STATES OF AMERICA) 4 April 1991 cited in the application	1-6,9, 16,19-26
X	see the whole document	27-31

-/--

III. DOCUMENTS CONSIDERED TO BE RELEVANT . (CONTINUED FROM THE SECOND SHEET)

Category °	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	WO,A,9 105 551 (THE UNITED STATES OF AMERICA) 2 May 1991 cited in the application	1-6,11, 12,19-26
X	see the whole document	27-33
Y	WO,A,9 205 149 (THE UNITED STATES OF AMERICA) 2 April 1992 cited in the application	1-6,10, 13-15, 19-26
X	see the whole document	27-32
Y	THE JOURNAL OF EXPERIMENTAL MEDICINE vol. 174, no. 4, 1 October 1991, pages 761 - 767 KWON, N.S. ET AL 'INHIBITION OF TUMOR CELL RIBONUCLEOTIDE REDUCTASE BY MACROPHAGE-DERIVED NITRIC OXIDE' see the whole document	1-21, 22-26, 33,34

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US 93/ 02922

B x I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
REMARK: Although claims 1-26 and 33 and 34 are directed towards a method of treatment of the human/animal body, the search has been carried out and based upon the alleged effects of the compounds.

2. ☒ Claims Nos.: 11-18, 33-34
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
In view of the large number of compounds which are theoretically defined by the formula of claims 11-18 the search had to be restricted for economic reasons. The search was directed towards these compounds specifically exemplified and the general concept of the application.

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searches without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON INTERNATIONAL PATENT APPLICATION NO.

US 9302922
SA 72738

This annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report.
The members are as contained in the European Patent Office EDP file on
The European Patent Office is in no way liable for these particulars which are merely given for the purpose of information. 06/07/93

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9009785	07-09-90	US-A-	4954526	04-09-90
		AU-B-	621470	12-03-92
		AU-A-	5192290	26-09-90
		EP-A-	0495776	29-07-92
		JP-T-	4505317	17-09-92
WO-A-9104022	04-04-91	US-A-	5039705	13-08-91
		AU-A-	6522690	18-04-91
		EP-A-	0491864	01-07-92
		JP-T-	5501402	18-03-93
		US-A-	5208233	04-05-93
WO-A-9105551	02-05-91	US-A-	5212204	18-05-93
		AU-A-	6621190	16-05-91
		CA-A-	2070388	19-04-91
		EP-A-	0501975	09-09-92
WO-A-9205149	02-04-92	US-A-	5155137	13-10-92
		AU-A-	8712391	15-04-92

EPO FORM P0479

For more details about this annex : see Official Journal of the European Patent Office, No. 12/82